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Deletion of the Ca*BIG1* Gene Reduces β-1,6-Glucan Synthesis, Filamentation, Adhesion, and Virulence in *Candida albicans*

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The human fungal pathogen *Candida albicans* is able to change its shape in response to various environmental signals. We analyzed the *C. albicans BIG1* homolog, which might be involved in β-1,6-glucan biosynthesis in *Saccharomyces cerevisiae*. *C. albicans BIG1* is a functional homolog of an *S. cerevisiae BIG1* gene, because the slow growth of an *S. cerevisiae big1* mutant was restored by introduction of *C. albicans BIG1*. CaBig1p was expressed constitutively in both the yeast and hyphal forms. A specific localization of CaBig1p at the endoplasmic reticulum or plasma membrane similar to the subcellular localization of *S. cerevisiae* Big1p was observed in yeast form. The content of β-1,6-glucan in the cell wall was decreased in the Ca*big1*Δ strain in comparison with the wild-type or reconstituted strain. The *C. albicans BIG1* disruptant showed reduced filamentation on a solid agar medium and in a liquid medium. The Ca*big1*Δ mutant showed markedly attenuated virulence in a mouse model of systemic candidiasis. Adherence to human epithelial HeLa cells and fungal burden in kidneys of infected mice were reduced in the Ca*big1*Δ mutant. Deletion of Ca*BIG1* abolished hyphal growth and invasiveness in the kidneys of infected mice. Our results indicate that adhesion failure and morphological abnormality contribute to the attenuated virulence of the Ca*big1*Δ mutant.

Candida albicans is an opportunistic fungal pathogen in humans and can cause either systemic or mucosal infection. In immunocompromised patients, this organism can progress to severe systemic invasion, leading to life-threatening circumstances (24, 25). *C. albicans* is a polymorphic fungus capable of converting its cell shape from budding yeast to filamentous form, including pseudohyphae and true hyphae. This morphological transition has been strongly associated with pathogenicity (9).

The cell wall of yeast is an elastic structure that provides physical protection and osmotic support and determines the shape of the cell (10, 17, 21, 28). Because of its major structural differences from human cells and its importance in fungal growth and virulence, cell wall biogenesis has long been focused on as a fascinating target for new antifungal agents. The mechanical strength of the wall is due mostly to the inner layer, which consists of β-glucan and chitin. The β-glucans are the main components in *C. albicans*, accounting for 50 to 60% by weight of the cell wall. Chitin is a minor (1 to 10%) but important constituent of the *C. albicans* cell wall, distributed at the septa between independent cell compartments, budding scars, and the ring around the constriction between mother cell and bud. The outer layer, which consists of heavily glycosylated mannoproteins emanating from the cell surface, is involved in cell-cell recognition events. Mannoproteins represent 30 to 40% of the total cell wall polysaccharide and determine the surface properties. Cell wall mannoproteins are covalently linked to the β-1,3-glucan–chitin network either indirectly through a β-1,6-glucan moiety or directly (10, 28). *C. albicans* hyphal cells contain twice as much chitin as the yeast cells do, whereas the increased levels of β-1,6-glucan and the decreased levels of mannoproteins in hyphal cells are due to the change in the growth temperature. *C. albicans* strains contain more than 20% β-1,6-glucan in the cell wall polysaccharides, while β-1,6-glucan constitutes about 12% of the wall polysaccharides in *Saccharomyces cerevisiae*. Furthermore, the fine structure of β-glucan differs between *S. cerevisiae* and *C. albicans*: the β-1,6-glucan polymer is less branched and contains fewer intrachain β-1,3 linkages in *C. albicans* than in *S. cerevisiae* (14).

The *S. cerevisiae* β-1,6-glucan is a highly branched polymer consisting of approximately 10% of the cell wall dry weight, with an average size of 350 residues. In *C. albicans*, β-1,6-glucan is particularly abundant, being present at almost double the amounts found in *S. cerevisiae* (14, 23). Based on genetic analyses of null strains, many genes involved in *S. cerevisiae* β-1,6-glucan biosynthesis have been identified; these include the *kre* mutants, which are resistant to the K1 killer toxin, which kills yeast following binding to a β-1,6-glucan-containing cell surface receptor (1, 5, 7). The proteins encoded by some *KRE* genes are located along the secretory pathway, including the endoplasmic reticulum (ER), Golgi apparatus, and plasma membrane. Kre5p is an ER protein that shares significant sequence similarity with UDP-glucose:glycoprotein glucosyltransferases and is epistatic to all other *KRE* genes that have been isolated to date (22). Many other gene products involved in β-1,6-glucan show no significant similarity to any protein with a known function. For *C. albicans*, there have also been reported several genes identified by homology with *S. cerevisiae* genes involved in β-1,6-glucan biosynthesis: Ca*KRE5* (14), Ca*KRE9* (18), Ca*KRE6*, and Ca*SKN1* (23). These reports de-

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TABLE 1. Strains used and constructed in this study

Strain or characteristic	Parent or characteristic	Genotype	Reference or source
S. cerevisiae			
BY4741		MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	6
big1Δ	BY47411	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 big1Δ::kanMX4*	31
BIG1YES	big1Δ	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 big1Δ::kanMX4* pYES2.0	This work
BIG1YES-SB	big1Δ	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 big1Δ::kanMX4* pYES2.0-Sc*BIG1*	This work
BIG1YES-CB	big1Δ	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 big1Δ::kanMX4* pYES2.0-Ca*BIG1*	This work
C. albicans			
CAI-4	SC5314	*ura3Δ::imm434/ura3Δ::imm434*	11
TUA4	CAI-4	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200*	15
TUA6	TUA5	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hig200/ARG4 RP10*::p3HA-ACT1	13
BIG101	TUA4	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200* Ca*big1Δ::hph200-URA3-hph200*/Ca*BIG1*	This work
BIG102	BIG101	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200* Ca*big1Δ::hph200-URA3-hph200*/Ca*big1Δ::ARG4*	This work
BIG103	BIG102	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200* Ca*big1Δ::hph200*/Ca*big1Δ::ARG4*	This work
BIG104	BIG103	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200* Ca*big1Δ::hph200*/Ca*big1Δ::ARG4 RP10*::p3HA-ACT1	This work
BIG105	BIG103	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200* Ca*big1Δ::hph200*/Ca*big1Δ::ARG4 RP10*::p3HA-ACT1-BIG1	This work
BIG1HA	TUA4	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200* Ca*BIG1*/Ca*BIG1*-3xHA	This work
BIG1GFP	TUA4	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 RP10*::pGFP-MET3-BIG1	This work

scribe the association of β-1,6-glucan biosynthesis with morphogenesis and virulence.

In *S. cerevisiae*, *BIG1* was first described as a multicopy suppressor of the synthetic lethality of the *rot1-1 rot2-1* double mutant; both mutations were identified by their ability to suppress the loss of *TOR2*, an essential phosphatidylinositol kinase homolog (4). The deletion of *BIG1* and *ROT1* leads to a remarkable reduction of the amount of β-1,6-glucan in the cell wall composition and thereby significant growth defects occur (2, 19, 26). Big1p is an N-glycosylated integral membrane protein with a type I topology and is located in the ER membrane (2). It is an indisputable fact that Big1p might play some role in β-1,6-glucan biosynthesis; however, the exact role of Big1p in the cell wall biosynthesis remains unknown. A homolog of *S. cerevisiae BIG1* has also been identified in the pathogenic fungus *C. albicans* (2). Here, we characterize *C. albicans BIG1* to determine the importance of Big1p to β-1,6-glucan synthesis and the impact that it has on hyphal growth and virulence. Big1p is expressed constitutively and is localized to ER or plasma membrane. Deletion of *BIG1* in *C. albicans* did not affect the rate of vegetative growth but did lead to defects in filamentation, adhesion, and virulence.

MATERIALS AND METHODS

Strains, growth conditions, and basic techniques. Table 1 lists the *S. cerevisiae* and *C. albicans* strains used in this study. Cells were grown in yeast extract-peptone-dextrose (YPD) (adjusted to pH 5.6; Qbiogene Inc.), SD-URA, or SD-AU (6.7 g liter^{-1} yeast nitrogen base without amino acids [Difco], 2% glucose, CSM-URA, or CSM-ARG-URA [Qbiogene Inc.]) with shaking to induce the yeast form or in YPD (adjusted to pH 7.2) plus 10% serum at 37°C with shaking to induce hyphae. For *S. cerevisiae*, yeast nitrogen base (YNB) agar [0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% $(NH_4)_2SO_4$, 2% galactose, 0.006% leucine, 0.002% histidine, 0.001% methionine, 0.0015% lysine, 2% agar] was used. The rate of growth was measured by determining the optical density at 660 nm with a TN-1506 biophotorecorder (Advantec, Japan). For determinations of filamentous growth on solid medium, strains were grown for 7 days at 37°C on 10% calf serum solidified with the addition of 2% agar.

Chlamydospore formation was performed as described by Martin et al. (20). Briefly, cells were grown overnight at 30°C in YPD medium and diluted 1:250 in sterile water. Three microliters of this suspension was spotted onto cornmeal-Tween agar (1.7% cornmeal agar [Nissui, Japan] plus 1% Tween 80). Coverslips were placed on top of the spots, and the plates were kept for 7 days at 25°C in the dark.

Escherichia coli XL1-Blue and cloning vector pUC19 (32) were used for DNA manipulation. General recombinant DNA procedures were performed as described by Sambrook and Russell (27). *C. albicans* was transformed by the method described by Umeyama et al. (29). An Applied Biosystems model 3100 automated capillary sequencer was used for nucleotide sequencing. Western analysis using anti-hemagglutinin (HA) or anti-PSTAIRE antibody, recognizing CaCdc28 or CaPho85 in *C. albicans*, was performed as described by Umeyama et al. (29). Microscopic observation was performed using a conventional fluorescence microscope (IX81; Olympus, Japan) equipped with a DP70 digital camera (Olympus, Japan).

Plasmid construction. For plasmids used in *S. cerevisiae*, a DNA fragment containing Sc*BIG1* or Ca*BIG1* was amplified using primers ScBIG1-N (5′-CGC GGATCCATTCTTTAATTATATCGA) and ScBIG1-C (5′-CCGGAATTCGT ATATAACGAACCATAA) or CaBIG1-N (5′-CCCAAGCTTGATAAAATGA GATTATTCGTCCTA) and CaBIG1-C (5′-CGGGATCCTTAATCTAATTTC TTATCGTCAGCA) with pRS426-BIG1 (2) or CAI-4 chromosomal DNA, respectively, as a template. Each DNA fragment was digested with BamHI and EcoRI or HindIII and BamHI and then cloned into the BamHI-EcoRI or HindIII-BamHI sites of pYES2.0 to generate pYES2.0-ScBIG1 or pYES2.0-CaBIG1, respectively.

A green fluorescent protein (GFP) sequence-containing XhoI-EcoRI DNA fragment of pGFP-ACT1 (29) was cloned into the XhoI-EcoRI sites of pFLAG-MET3 (30) to generate pGFP-MET3. For plasmids containing the Ca*BIG1* gene, a DNA fragment containing Ca*BIG1* was PCR amplified using two primers, BIG1-N (5′-GCCGGATCCAGGATGAGATTATTCGTCCTACTAG) and BIG1-C (5′-G GCGCATGCATCTAATTTCTTATCGTCAGC), with TUA4 chromosomal DNA as a template; digested with BamHI and SphI; and then cloned into the BamHI-SphI sites of p3HA-ACT1 (29) and pGFP-MET3 to generate p3HA-BIG1 and pGFP-BIG1, respectively. The nucleotide sequences of the cloned fragments were confirmed.

Strain construction. Gene disruption was performed using a method similar to that described by Hanaoka et al. (13). Briefly, two fragments, disBIG1-A and disBIG1-B, were amplified with primers disBIG1-1 (5′-ACATGGTGAATCAGTAT GAGCACC) and disBIG1-2 (5′-GTCGTGACTGGGAAAACCCTGGCGATCT CAAGAAACGAACTTTTGAGC) and disBIG1-3 (5′-CCTGTGTGAAATTGTT ATCCGCTCCAAAATATAGTGATGGGTCCAAAC) and disBIG1-4 (5′-TCAA ATTGTGTTGACAGTGGGACC), respectively, and used as flanking homology

regions for a gene disruption cassette. The PCR-amplified disruption cassette containing an *hph200-URA3-hph200* or *ARG4* marker was transformed into the TUA4 *arg4*$^-$ *ura3*$^-$ strain. Finally, both alleles of the Ca*BIG1* locus were replaced with *hph200* and *ARG4*, yielding strain BIG103. For a complementation test, the empty vector p3HA-ACT1 or the plasmid p3HA-ACT1-BIG1 was introduced into BIG103, yielding strain BIG104 or BIG105, respectively.

To obtain a strain in which CaBig1p is tagged at the C terminus with a triple repeat of the HA tag, we used p3HA-ACT1 (29) as a template for PCR-mediated transformation. A DNA cassette for integration was amplified using the primers iBIG1-5′ (5′-AAGATCATTTCCTTCTTCAATTACTTGAAACAAAAAATAATACAAAAGAAACAACAAAAATCGAAGCGAGGTATTATTGCTGACGATAAGAAATTAGATTGCAGGCTCGAGGGTGCATGC) and iBIG1-3′ (5′-TAGCAAAAACTAGTTATAGAAAGTGTATATAAACATGAGTATGAATTTTTCTTAAAACATTCTAACAAAAAGCATTGCCAAACAACATTATTCTCTGAGCGGATAACAATTTCACACAGG) and introduced into TUA4. After selection on SD-URA plates, nucleotide sequencing confirmed that the colony PCR-amplified DNAs encoding the corresponding proteins were tagged correctly.

Quantification of cell wall component. Alkali-insoluble β-glucan levels were determined as described previously (16). Cells were cultured in 500-ml flasks containing 100 ml YPD plus 0.6 M sorbitol (for *S. cerevisiae*) or YPD with or without 10% serum (for *C. albicans*), harvested by centrifugation, and washed. The cells were broken with glass beads on ice, and the cell wall fraction was obtained by centrifugation at 2,600 × *g* for 15 min. Alkali-insoluble β-glucans were extracted three times in 3% (wt/vol) NaOH at 75°C for 1 h. The pellet was washed three times, resuspended in 0.01 M Tris-HCl (pH 7.5) containing β-1,3-glucanase (Zymolyase 100T, 1.0 mg/ml), and incubated overnight at 37°C. After dialysis, the β-1,6-glucan was obtained. The total alkali-insoluble glucan level was measured as the hexose content before dialysis. The alkali-insoluble β-1,3-glucan level was calculated by subtraction of the β-1,6-glucan content from the total glucan content. Chitin was quantified as described previously (8), except that we used β-glucuronidase instead of cytohelicase.

Preparation of total cell lysates and solubility test of CaBig1p protein. Cells were collected and disrupted with glass beads in NP-40 buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1% NP-40) by use of a bead shocker (Yasui Kikai, Japan). After centrifugation at 10,000 × *g* for 10 min, the supernatant was extracted for Western analysis. To demonstrate membrane association, aliquots of total cell lysate extracted with lysis buffer (50 mM Tris-HCl [pH 8], complete protease inhibitor cocktail [Roche]) were separately treated with 0.6 M NaCl, 0.1 M Na_2CO_3, 1.6 M urea, or 0.5% Triton X-100 at 4°C for 30 min and subsequently centrifuged at 30,000 × *g* at 4°C for 30 min. The supernatant was withdrawn and the pellets were resuspended in a volume of lysis buffer equal to that of the supernatant.

Adherence assay. The adherence assay was conducted fundamentally as previously described (3). Briefly, *C. albicans* cells from an overnight culture in YPD medium at 30°C were washed and diluted in Dulbecco's modified Eagle's medium containing 10% calf serum. A suspension containing 10^5 CFU/ml was then preincubated for 1 h at 37°C. HeLa cells were grown to confluence in Dulbecco's modified Eagle's medium containing 10% calf serum at 37°C (5% CO_2) in 96-well culture plates. They were then washed once with 1× PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4), and then 100 μl of the *Candida* cell suspension was added. After incubation at 37°C for 30 min, the nonadherent *Candida* cells were washed away three times with PBS. The adherent *Candida* cells were released by lysing the HeLa cells with sterile water. The recovered *Candida* cells were plated onto YPD. After incubation at 30°C for 24 h, the number of CFU was determined.

Animal study. For each group, five male CD-1 (ICR) mice of 4 weeks of age (Charles River, Japan) weighing approximately 21 to 25 g were inoculated with 10^6 CFU by intravenous injection. Survival curves were calculated using the Kaplan-Meier method and then compared by use of the log rank test. A *P* value of less than 0.05 was considered significant. To quantify the colony-forming *C. albicans* units in the kidneys, three mice were euthanized by CO_2 5 days after injection, after which the organs were homogenized and plated onto an SD-AU medium for colony counting. For histopathological examination of the kidney, the kidneys were fixed in 10% phosphate-buffered formalin, embedded, sectioned, and stained with periodic acid-Schiff (PAS) stain.

RESULTS

***C. albicans BIG1* encodes a functional homolog of *S. cerevisiae* Big1p.** A *C. albicans* Ca*BIG1* homolog (CaO19.2334 and CaO19.9870) encodes a protein of 322 amino acids, showing 29% identity to its *S. cerevisiae* counterpart (2). In this paper, we prefix a gene name with "Sc" or "Ca" to avoid confusing *S. cerevisiae* and *C. albicans* genes. In order to determine whether *C. albicans* CaBig1p is a functional homolog of *S. cerevisiae* ScBig1p, *S. cerevisiae* Sc*big1*Δ cells were transformed with plasmid pYES2.0-CaBIG1 containing the *C. albicans* Ca*BIG1* gene under control of the *GAL1* promoter. The Sc*big1*Δ mutant has a growth defect and requires osmotic support (2). Figure 1A shows that *C. albicans* Ca*BIG1* complemented the growth deficiency of *S. cerevisiae* Sc*big1* mutants on YNB agar plates. We then analyzed the cell wall composition and found that *C. albicans* Ca*BIG1* increased

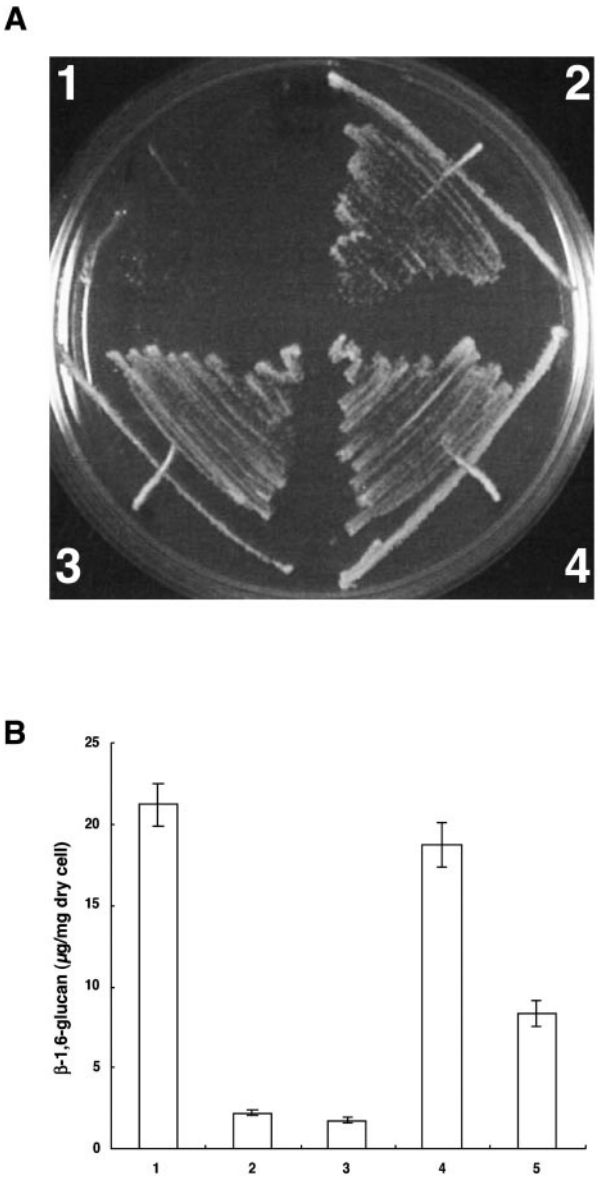

FIG. 1. Complementation of the growth phenotype (A) and the β-1,6-glucan defect (B) of the *S. cerevisiae* Sc*big1*Δ null mutant by the *C. albicans* Ca*BIG1* gene. (A) The Sc*big1*Δ strain harboring pYES2.0 empty vector (1), pYES2.0-ScBIG1 (2), and pYES2.0-CaBIG1 (3 and 4) was streaked on YNB agar. (B) The wild type (1), an Sc*big1*Δ mutant (2), and the same mutant harboring empty vector (3), Sc*BIG1* (4), or Ca*BIG1* (5) on multicopy vector pYES2.0 were scored for the amount of β-1,6-glucan. The data shown represent the results of at least three experiments. Error bars represent standard deviations.

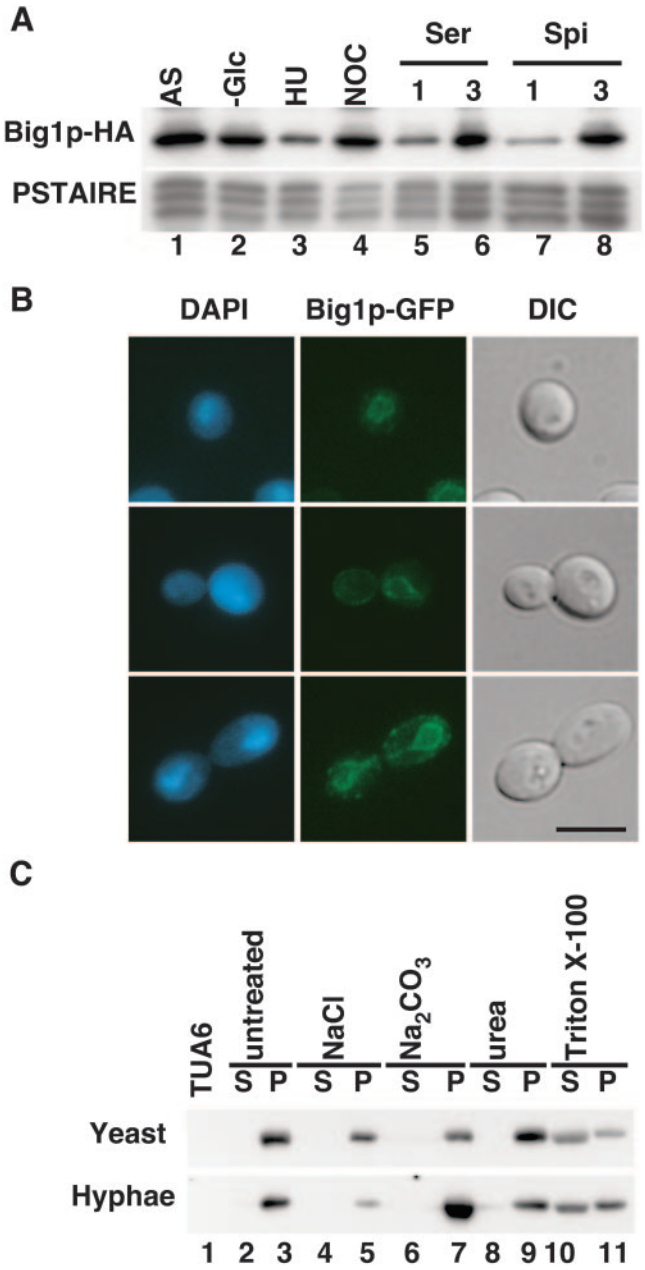

FIG. 2. CaBig1p localized in the ER and plasma membrane of *C. albicans* yeast cells. (A) Western blotting for the detection of CaBig1p-3xHA. TUA4 cells expressing CaBig1p tagged with three tandem repeats of HA (CaBig1p-3xHA) from its native promoter were used. Each lane was processed using a total cell extract with the following culture conditions: AS, asynchronous cells cultured for three hours at 30°C; -Glc, unbudded cells collected by YNB medium (without glucose); HU, synchronized cells with 0.1 M hydroxyurea; NOC, synchronized cells with 20 μg/ml nocodazole; Ser, hyphal cells cultured in YPD containing 10% serum for 1 or 3 hours as indicated; Spi, hyphal cells cultured in Spider medium for 1 or 3 hours as indicated. Western blotting using anti-PSTAIRE antibody was performed as a loading control. (B) Fluorescence microscopy for the detection of CaBig1p-GFP. TUA4 cells expressing GFP-tagged CaBig1p were grown overnight at 30°C, inoculated into fresh YPD (pH 5.6) medium, fixed in 3% formaldehyde, and viewed under a fluorescence microscope and differential interference contrast (DIC) optics. DAPI (4′,6′-diamidino-2-phenylindole)-stained cells, CaBig1p-GFP, and differential interference contrast images are shown. Bar, 5 μm. (C) Solubility test of CaBig1p-3xHA. Cell lysate from yeast or hyphal forms of the wild-type TUA6 (lane 1) or wild-type cells expressing CaBig1p-3xHA was left untreated (lanes 2 and 3) or was treated with NaCl (lanes 4 and 5), Na_2CO_3 (lanes 6 and 7), urea (lanes 8 and 9), or Triton X-100 (lanes 10 and 11) and subsequently centrifuged and separated into pellet (P) and supernatant (S) fractions.

the β-1,6-glucan content of the *S. cerevisiae* Sc*big1*Δ cells (Fig. 1B). There was a significant difference between Sc*big1*Δ harboring the pYES2.0 empty vector and that harboring pYES2.0-CaBIG1, with a P value of <0.005, whereas Ca*BIG1* did not restore the reduced content of β-1,6-glucan as much as Sc*BIG1* did. These results suggest some functional conservation between the Big1p homologs of *C. albicans* and *S. cerevisiae*.

Constitutive expression and localization of CaBig1p. To determine whether CaBig1p is posttranslationally modified like N glycosylation of ScBig1p and whether CaBig1p expression alters during the cell cycle or in response to hyphal induction, CaBig1p was tagged with three tandem repeats of HA at the C terminus, and protein samples were prepared from yeast or hyphal cells for Western blot analysis. Yeast cells were cultured in YPD, pH 5.6, at 30°C with or without a cell cycle toxin such as hydroxyurea or nocodazole. Hyphal growth was induced in YPD, pH 7.2, containing 10% serum or Spider medium at 37°C. There was no significant difference in signal strengths or mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis assays between the conditions (Fig. 2A).

To examine CaBig1p localization in *Candida* cells, a fragment encoding the GFP was fused in frame to the 3′ end of Ca*BIG1*. The fusion construct was placed under control of the *MET3* promoter in pGFP-MET3 plasmid containing *RP10*/*URA3*. Yeast cells containing CaBig1p-GFP showed ring-like GFP fluorescence surrounding the nucleus, which implies localization in the ER membrane, and dot-like fluorescence in the plasma membrane (Fig. 2B), while the wild-type cells, which contain no GFP fusion protein, showed almost no fluorescent signals under the same circumstances of fluorescence, as in the case of CaBig1p-GFP (data not shown). However, CaBig1p-GFP was not localized in specific areas of the hyphal cells under our laboratory conditions (data not shown). Thus, these results suggested that CaBig1p is associated with the ER and plasma membrane in a manner similar to that of its homolog in *S. cerevisiae* (2).

In cell fractionation experiments, we further examined whether CaBig1p is localized in cellular membranes. Cell lysates of CaBig1p-3xHA-expressing cells were treated with different agents that solubilize peripheral membrane proteins (12). As shown in Fig. 2C, 1% Triton X-100 solubilized CaBig1p partially from the pellet fraction (lanes 10 and 11), while neither NaCl, urea, nor Na_2CO_3 did (lanes 2 to 9), suggesting that CaBig1p is an integral membrane protein. Along with the GFP data, these results indicate that CaBig1p was localized in the ER membranes and/or plasma membranes of the *C. albicans* yeast cells.

Cell wall component of *C. albicans* cells lacking CaBig1p. To study the relationship of the cell wall component and Ca*BIG1*, we constructed strain BIG103, which lacked both alleles of the Ca*BIG1* locus, by PCR-based gene disruption (13). Strain BIG103 was transformed with the empty p3HA-ACT1 vector

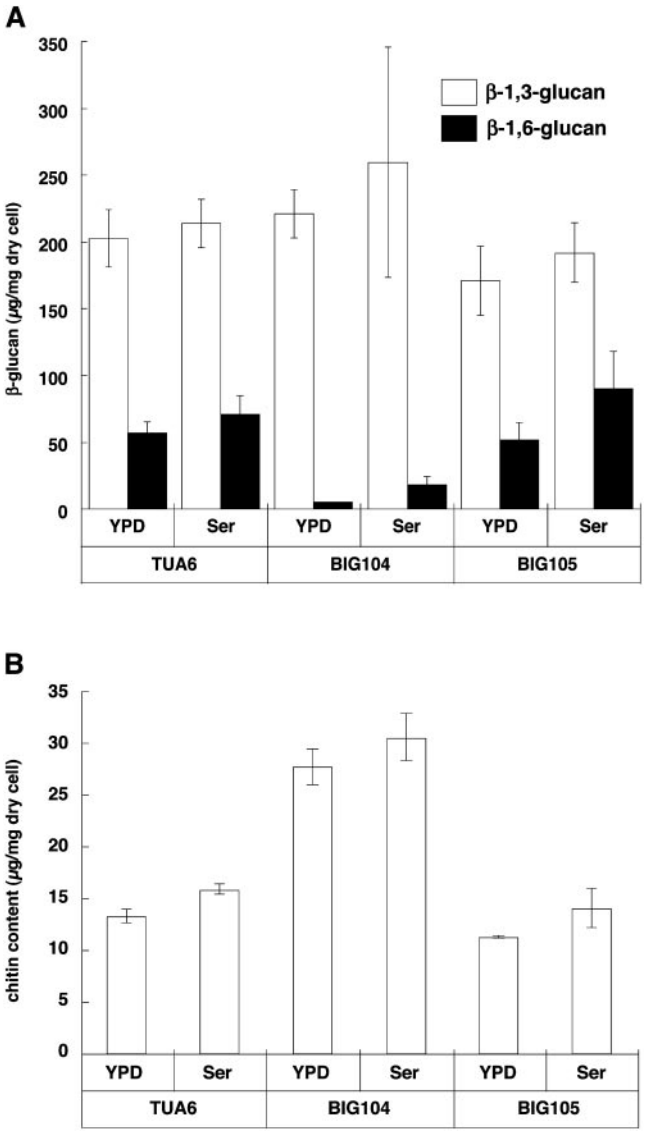

FIG. 3. Repression of β-1,6-glucan biosynthesis in C*abig1*Δ mutants. Alkali-insoluble β-1,6-glucans (closed bars) and β-1,3-glucans (open bars) (A) and chitin (B) were measured. Cells, including wild-type (TUA6), C*abig1*Δ (BIG104), and reconstituted (BIG105) strains, were grown for 4 h at 30°C in YPD, pH 5.6 (YPD) or at 37°C in YPD, pH 7.2, containing 10% serum (Ser). The data shown represent the results of at least three experiments. Error bars represent standard deviations.

to generate BIG104. A reconstituted strain, BIG105, was obtained by the introduction of p3HA-BIG1 into a C*abig1*Δ null mutant. Both the null mutant BIG104 and the reconstituted strain BIG105 have a single copy of *URA3* at the *RP10* locus. The amounts of whole-cell alkali-soluble β-1,6-glucan in the wild-type, C*abig1*Δ, and reconstituted strains were compared by measuring alkali-insoluble β-glucan treated or untreated with the β-1,3-glucan digestive enzyme, zymolyase 100T. As shown in Fig. 3A, alkali-insoluble β-1,6-glucan in *C. albicans* C*abig1*Δ cells was almost undetectable and was restored to its normal level in the reconstituted strain, suggesting that Ca*BIG1* is required for β-1,6-glucan biosynthesis. Moreover, an increased level of chitin seen in the C*abig1*Δ mutant (Fig. 3B) implies that this increase may compensate for the defect in β-1,6-glucan biosynthesis. In contrast, no distinguishable difference in β-1,3-glucan levels was observed for the parent, the C*abig1*Δ strain, and the revertant (Fig. 3A), unlike what was seen for *S. cerevisiae* Sc*big1*Δ, which showed a significant increase in the level of β-1,3-glucan (2). Thus, the deletion of the Ca*BIG1* gene significantly affected β-1,6-glucan biosynthesis.

***C. albicans* CaBig1p is required for yeast morphology.** The ability to generate a viable C*abig1*/C*abig1* null mutant strain indicates that Ca*BIG1* is not an essential gene in *C. albicans*. Deletion of the *S. cerevisiae* Sc*BIG1* gene is lethal, but the gene was partially restored by adding sorbitol to the medium as osmotic support (2). Unlike what was seen for the *S. cerevisiae* Sc*big1* mutant, growth of the wild-type, C*abig1*Δ, and reconstituted strains showed no difference in doubling time in YPD medium (data not shown).

Disruption of the *C. albicans BIG1* gene generated a slightly abnormal cell shape. When vegetatively grown in liquid YPD medium, null mutant cells were slightly elongated or distorted and tended to aggregate (Fig. 4). A large aggregate of C*abig1*Δ cells could be easily separated into chains of two to eight cells by vigorous pipetting, indicating that the aggregation was caused not only by cell-to-cell attachment but also by a partial cytokinesis defect. However, more than 80% of cells in stationary phase, obtained by overnight culture in YPD medium at 30°C, showed budded or unbudded single cells (data not shown). Unlike *C. albicans* cells lacking the Ca*KRE5* gene (14), which reduces the levels of β-1,6-glucan synthesis and shows aberrant shape, the average sizes of the cells and vacuoles of the C*abig1* disruptant were the same as were those of the wild type.

***C. albicans* CaBig1p is required for hyphal morphogenesis.** *C. albicans* cells lacking CaKre5p (14) or CaKre9p (18), both of which are involved in β-1,6-glucan synthesis, showed deficient filamentation. In order to examine whether the absence of *C. albicans* CaBig1p affected hyphal morphogenesis in *C. albicans*, we investigated hyphal morphogenesis of the wild-type, C*abig1*Δ/C*abig1*Δ, and reconstituted strains under several conditions on both solid and liquid media. Initially, we investigated possible roles of Ca*BIG1* in hyphal growth in liquid media. We found that unlike the *C. albicans* C*akre5*Δ/C*akre5*Δ or C*akre9*Δ/C*akre9*Δ mutant, the C*abig1*Δ homozygous mutant underwent hyphal transition at 37°C in liquid YPD plus 10% calf serum, but its hyphal cells tended to be slightly distorted compared to those of the wild-type or reconstituted strain (Fig. 4). In addition, the C*abig1*Δ cells grown at 37°C in liquid Spider medium showed a pseudohyphal cell shape. The defects in filamentation were partially restored by the addition of 1.25% *N*-acetylglucosamine to the medium, as in C*akre5*Δ cells (14), and also by the addition of 0.6 M sorbitol as an osmotic support (data not shown).

Next, we investigated the effects of Ca*BIG1* deletion on solid agar medium. Cells were grown overnight at 30°C in YPD liquid medium, and 10^6 cells were spotted on Spider agar medium or agar plus 10% serum medium. The homozygous C*abig1*Δ/C*abig1*Δ mutant did not form lateral hyphae at 30°C on Spider medium and formed only short filamentation at 37°C on serum agar medium. The addition of 1.25% *N*-acetylglucosamine to the Spider medium slightly restored the deficiency in filamentation (Fig. 5), as in the liquid medium, but had no effect on serum agar (data not shown). Combining these results demonstrated that the deletion of *C. albicans* Ca*BIG1* reduced

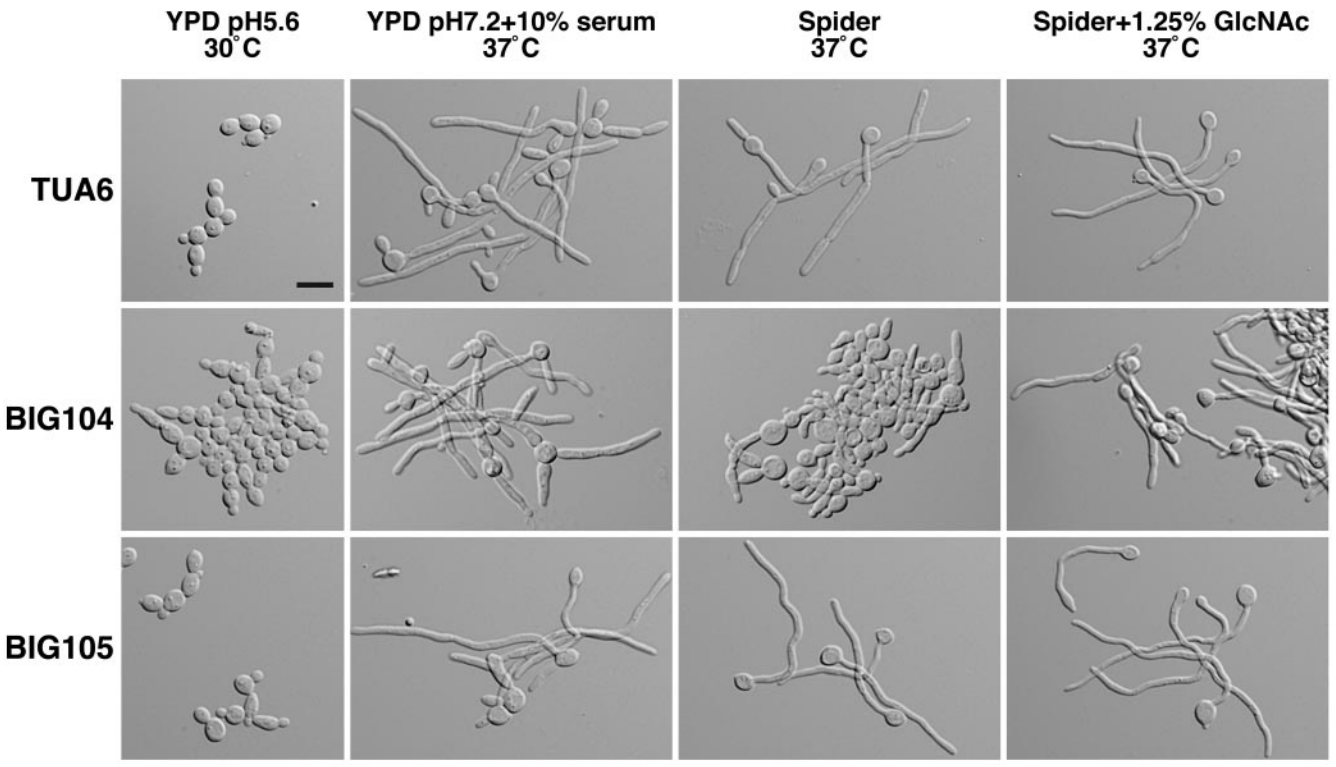

FIG. 4. Morphology of *C. albicans* strains in liquid medium. Cells, including wild-type (TUA6), Ca*big1*Δ (BIG104), and reconstituted (BIG105) strains, were grown for 4 h under the conditions indicated above. Bar, 10 μm.

the ability to elongate hyphal cells, but the ability to undergo yeast-to-hyphae transition remained.

Ca*big1*Δ produces a chlamydospore at the tip of short hyphae. The chlamydospore is a distinctive morphological feature of the fungal pathogen *C. albicans*. In order to examine the association between β-1,6-glucan biosynthesis and chlamydospore formation, cornmeal agar was used to compare the phenotypes of the wild-type, Ca*big1*Δ mutant, and reconstituted strains. The Ca*big1*Δ mutant apparently formed chlamydospores accompanied by short hyphae close to the edge of the colony, whereas the wild-type and reconstituted strains formed normal chlamydospores at the end of long hyphae (Fig. 6).

Deletion of Ca*BIG1* in *C. albicans* reduces adherence to HeLa cells. Herrero et al. (14) demonstrated that the ability of the Ca*KRE5* mutant to adhere to epithelial cells was reduced. To study the Ca*BIG1* gene in relation to adherence, we examined the abilities of the wild-type, the disruptant, and the reconstituted strains to adhere to a monolayer of human HeLa cells. Cells were

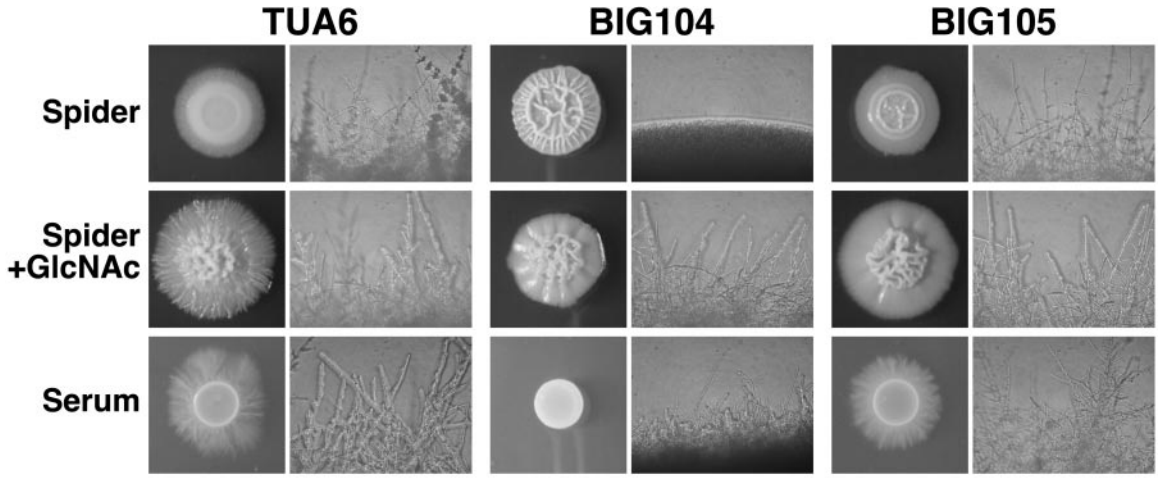

FIG. 5. Morphology of *C. albicans* strains on solid agar medium. Cells, including wild-type (TUA6), Ca*big1*Δ (BIG104), and reconstituted (BIG105) strains, were grown overnight at 30°C. Then, 10^6 cells were spotted onto the indicated agar plate and grown for 7 days at 30°C on Spider medium and at 37°C on agar medium containing 10% serum. Photographs of the colony edge were taken by phase-contrast microscopy at ×20 magnification.

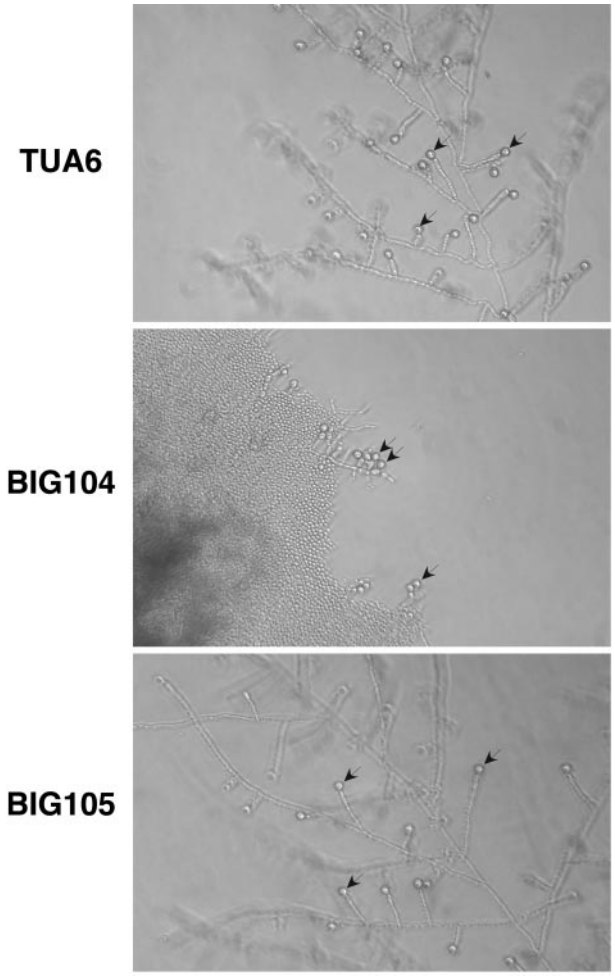

FIG. 6. Phenotypes of the wild-type (TUA6), C*abig1*Δ (BIG104), and reconstituted (BIG105) strains grown in cornmeal agar under glass coverslips for 7 days at 25°C in the dark. The chlamydospores, several of which are indicated by arrows, are the large round refractile cells at the end of the hyphae. Magnification, ×10.

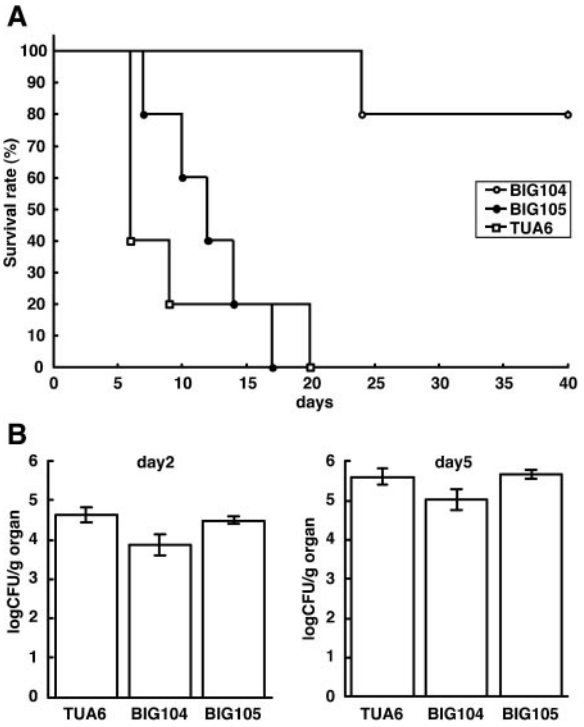

FIG. 7. The C*abig1*Δ mutant exhibits markedly reduced virulence. (A) *C. albicans* cells of wild-type (TUA6), C*abig1*Δ (BIG104), and reconstituted (BIG105) strains were grown overnight in YPD. Each mouse was injected via the tail vein with 10^6 CFU and monitored for death for 40 days. (B) Fungal burden of the kidneys in infected mice ($n = 3$).

cultured overnight in YPD medium at 30°C; this showed that more than 80% in culture were single cells. Yeast cells (10^5 CFU/ml) were preincubated in 10% serum for 1 h at 37°C, resulting in short hyphae, and then were placed on a HeLa monolayer for 1 h. Adherence was determined by comparison with the CFU counts of the wild-type TUA6 cells attached to a monolayer of HeLa cells. The adherences of the disruptant BIG104 and the reconstructed strain BIG105 were 45.7% ± 4.7% and 93.5% ± 9.8% (mean ± standard deviation), respectively, with a P value of <0.005 compared to that of the wild type.

Ca*BIG1* is required for virulence. Using the mouse systemic candidiasis model, we found that 80% of the mice infected with 10^6 CFU cells of C*abig1*Δ remained alive 40 days postinfection, whereas the same inoculum of the wild-type or of the reconstructed strain killed all infected mice within 20 days (Fig. 7A). To determine whether the reduced virulence of the C*abig1*Δ mutant against the mouse model infection correlated with reduced levels of tissue infection, we determined the fungal burden on the kidneys. The number of *Candida* cells colonized in the kidneys of mice infected with the C*abig1*Δ mutant was 1 order of magnitude below that of those infected with the wild-type strain or with the Ca*BIG1* revertant (Fig. 7B). To investigate the morphology of the C*abig1*Δ cells in the kidney of systemically infected mice, mice injected via tail vein with 10^6 cells were killed after 2 days, and the kidneys were removed for histopathological examination. PAS-stained kidney sections showed that the C*abig1*Δ mutant did not form hyphae in the infected lesion, whereas distinguishable hyphae penetrating into tissue formed in the wild-type and reconstituted strains (Fig. 8), demonstrating that CaBig1p is required for filamentation in mice. These results show that Ca*BIG1* is required for *C. albicans* virulence and support the concept that hyphal morphogenesis and adherence are important for the pathogenesis of this organism.

DISCUSSION

We have characterized a *C. albicans* gene that is the sole homolog of the *BIG1* gene of *S. cerevisiae*. A homozygous C*abig1*Δ gene disruptant was constructed by combining a PCR-amplified deletion cassette with a split Ura-blaster technique and analyzed with respect to cell wall composition, morphology, adherence, and virulence. The amino acid sequence identity between ScBig1p and CaBig1p is 29% (2), and the growth defect of the S*cbig1*Δ mutant was partially complemented by the expression of the Ca*BIG1* gene, indicating that CaBig1p is a functional homolog of ScBig1p. However, the effect caused by deletion of the Ca*BIG1* gene differs substantially from that of Sc*BIG1*. A major phenotypic difference is growth rate. *S. cerevisiae big1* disruptants failed to grow, whereas addition of an osmotic reagent, such as sorbitol,

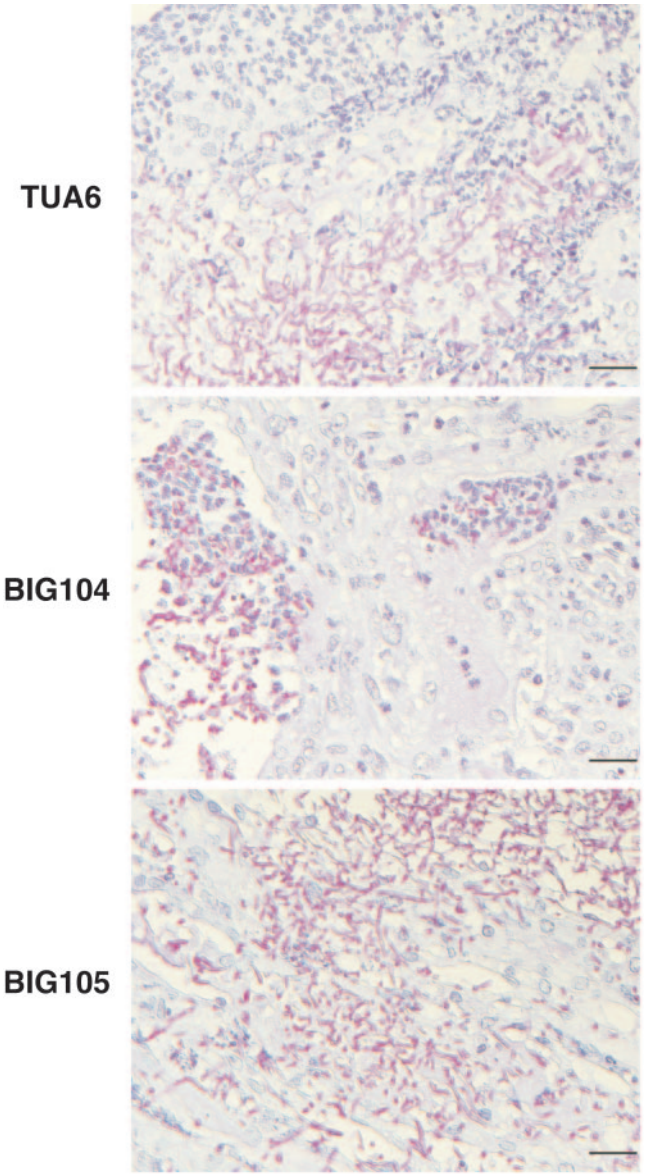

FIG. 8. Histopathological analysis of the kidneys of *C. albicans*-infected mice. The kidneys of the infected mice were removed at day 2 postinfection, fixed, sectioned, and PAS stained. Bar, 20 μm.

slightly restored growth (2). In contrast, Ca*big1*Δ cells grew normally, like the wild-type cells, indicating that Ca*BIG1* is not essential for *C. albicans* vegetative growth. In addition, the growth rate of the *S. cerevisiae* Sc*KRE5* deletion mutant is much slower than that of the *C. albicans* Ca*kre5*Δ mutant; Kre5p is considered a glucosyltransferase that is involved in the initiation of β-1,6-glucan synthesis (14). The differences in the roles of these gene products between *S. cerevisiae* and *C. albicans* might be derived from the characteristic that the cell wall composition in *C. albicans* is different from that in *S. cerevisiae* (14, 23).

The protein functions of *C. albicans* CaBig1p and of ScBig1p in *S. cerevisiae* remain unknown. Since deletion of the *BIG1* gene in both organisms leads to a defect in β-1,6-glucan biosynthesis, Big1p must be involved either directly or indirectly in cell wall biogenesis. What is the function of the Big1p protein molecule? As no homologs other than those in a family of budding yeast have thus far been identified, the function of Big1p could not be deduced from the similarity to other known proteins. Both CaBig1p and ScBig1p localize to the ER integral membrane, while most other known factors that affect β-1,6-glucan synthesis have been localized along the protein secretory pathway (28). Based on these facts, multiple events within the ER-Golgi apparatus are thought to be required for the proper biosynthesis of the β-1,6-glucan polymer. Taken together, these results indicate that Big1p might play a role as an enzyme that catalyzes the addition or modification of the sugar chain or as an adaptor protein that captures such enzymes. At present, we consider CaBig1p to be an adaptor protein rather than a functional enzyme because neither CaBig1p nor ScBig1p contains any amino acid motifs associated with carbohydrate-active enzymes. Using a tandem affinity purification technique, we are currently investigating whether CaBig1p and ScBig1p have binding partner proteins (15).

The results presented here revealed that CaBig1p is required for β-1,6-glucan biosynthesis and filamentation in *C. albicans*. Four *C. albicans* genes, Ca*SKN1* (23), Ca*KRE5* (14), Ca*KRE6* (23), and Ca*KRE9* (18), have been identified based on their homology with *S. cerevisiae* β-1,6-glucosylation, and their functions have been elucidated by null mutant analysis. The homozygous Ca*KRE9* and Ca*KRE5* deletion mutants and the heterozygous Ca*KRE6* deletion mutant showed 0, 20, and 60% reductions of the level of β-1,6-glucan synthesis, respectively. Although the Ca*kre6*Δ mutant, which has no severe reduction in β-1,6-glucosylation, shows no significant difference in morphology, no filamentation was observed in the Ca*kre5*Δ and Ca*kre9*Δ gene disruptants. Likewise, our studies demonstrated that only a small amount of β-1,6-glucan was detected in Ca*big1*Δ. However, the observation of Ca*big1*Δ mutant morphology was slightly different from that of the Ca*kre5*Δ or Ca*kre9*Δ mutant morphology. Under all conditions tested, no germ tube formation was observed in the Ca*kre5*Δ or Ca*kre9*Δ mutant, whereas the ability to form germ tubes partially remained in serum medium in the Ca*big1*Δ mutant. Thus, the Ca*big1*Δ mutant did not affect filamentation as severely as the other mutants did, despite the low level of β-1,6-glucan synthesis. Perhaps CaKre5p and CaKre9p may be functionally epistatic to CaBig1p. Thereby, the function of CaBig1p might be restricted to β-1,6-glucan synthesis. CaKre5p might be involved in protein sorting via glucosylation, as are other functions besides cell wall biogenesis. The reason for this might be that the Ca*kre5*Δ cells showed large-vacuole morphology, probably because of the accumulation of polypeptides that failed to acquire a mature conformation and are degraded in this organelle (14).

Why did the β-1,6-glucosylation-defective cells fail to form hyphae? We propose two answers to this question. First, β-1,6-glucan synthesis itself is indispensable for hyphal morphogenesis but not for yeast growth. The apical growth of hyphae requires rapid reshaping and expansion of the cell wall at the apical tip. Second, proteins including CaKre5p or CaBig1p play a pivotal role in directing proteins which are required for hyphal formation and/or β-1,6-glucan synthesis to the site where the cell wall is actively assembled. The possibility remains that CaBig1p catalyzes β-1,6-glucosylation directly.

Deletion of Ca*BIG1* leads to reduced pathogenicity in a mouse model of systemic infection. The attenuated virulence

of Ca*big1*Δ can be accounted for by two major in vivo properties: the inability to change shape to the filamentous form in the kidney and the reduced fungal burden. Loss of morphological transition of Ca*big1*Δ in the kidneys of infected mice, shown by histological observation, is consistent with the phenotype of the disruptant either on a solid medium or in a liquid medium inducing filamentation. The decreased number of *C. albicans* Ca*big1*Δ cells colonized in the kidney is supported by the adhesion experiment, in which the ability of the Ca*big1*Δ cells to adhere to HeLa cells is less than half that of the wild type. The Ca*KRE5* deletion mutant also showed similar virulence properties (14). Although there are only two reports, including our study, that indicate that a defect in β-1,6-glucan synthesis leads to avirulence, animal studies with other *C. albicans* mutants involved in β-1,6-glucan synthesis should explore all that is known about pathogenicity-associated cell wall biogenesis.

To conclude, the deletion of Ca*BIG1* in *C. albicans* leads to a decreased amount of β-1,6-glucan in the cell wall composition, thereby resulting in defects in filamentation, adhesion, and pathogenicity. Since no other sequences similar to Big1p have been identified in vertebrate animals so far, the β-1,6-glucan synthetic pathway, including Big1p, could be a good target for the development of novel antifungal drugs.

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